Received: 5 May 2020 | Accepted: 14 May 2020

DOI: 10.1002/rth2.12403

ORIGINAL ARTICLE

rpth research & practice in thrombosis & haemostasis

Liver fibrosis is driven by protease-activated receptor-1 expressed by hepatic stellate cells in experimental chronic liver injury

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Funding information
This work was supported by grants from the National Institutes of Health (R01ES017537 to JPL) (T32ES007255, F32 DK121423 to LGP), and additional support from the US Department of Agriculture (USDA) National Institute of Food and Agriculture (to JPL). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Environmental Health Sciences or USDA.

Handling Editor: Prof. Yotis Senis

Abstract

Background: Blood coagulation protease activity is proposed to drive hepatic fibrosis through activation of protease-activated receptors (PARs). Whole-body PAR-1 deficiency reduces experimental hepatic fibrosis, and *in vitro* studies suggest a potential contribution by PAR-1 expressed by hepatic stellate cells. However, owing to a lack of specific tools, the cell-specific role of PAR-1 in experimental hepatic fibrosis has never been formally investigated. Using a novel mouse expressing a conditional PAR-1 allele, we tested the hypothesis that PAR-1 expressed by hepatic stellate cells contributes to hepatic fibrosis.

Methods: PAR-1$^{flox/flox}$ mice were crossed with mice expressing *Cre* recombinase controlled by the lecithin retinol acyltransferase (LRAT) promoter, which induces recombination in hepatic stellate cells. Male PAR-1$^{flox/flox}$/LRATCre and PAR-1$^{flox/flox}$ mice were challenged twice weekly with carbon tetrachloride (CCl_4, 1 mL/kg i.p.) for 6 weeks to induce liver fibrosis.

Results: PAR-1 mRNA levels were reduced (>95%) in hepatic stellate cells isolated from PAR-1$^{flox/flox}$/LRATCre mice. Hepatic stellate cell activation was evident in CCl_4-challenged PAR-1$^{flox/flox}$ mice, indicated by increased α-smooth muscle actin labeling and induction of several profibrogenic genes. CCl_4-challenged PAR-1$^{flox/flox}$ mice displayed robust hepatic collagen deposition, indicated by picrosirius red staining and type I collagen immunolabeling. Notably, stellate cell activation and collagen deposition were significantly reduced (>30%) in PAR-1$^{flox/flox}$/LRATCre mice. Importantly, the reduction in liver fibrosis was not a consequence of reduced acute CCl_4 hepatotoxicity in PAR-1$^{flox/flox}$/LRATCre mice.

Conclusions: The results constitute the first direct experimental evidence that PAR-1 expressed by stellate cells directly promotes their profibrogenic phenotype and hepatic fibrosis *in vivo*.

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KEYWORDS

carbon tetrachloride, collagen, hepatic stellate cells, liver fibrosis, PAR-1, receptor, thrombin

Essentials

- Protease-activated receptor (PAR)-1 links coagulation protease activity to chronic liver damage.
- The PAR-1-expressing hepatic cell type contributing to liver fibrosis is not known.
- Hepatic stellate cell–specific PAR-1 deficiency does not affect acute hepatotoxicity.
- Stellate cell PAR-1 drives their profibrotic phenotype and hepatic fibrosis.

1 | INTRODUCTION

Experimental and clinical evidence suggests a role for activation of the blood coagulation cascade in the pathogenesis of chronic liver disease. Plasma levels of biomarkers indicative of thrombin generation are increased in patients with chronic liver disease, and activation of blood coagulation is a conspicuous feature of experimental liver injury.[1] Administration of anticoagulant drugs or genetically inhibiting coagulation cascade activation reduces liver fibrosis in multiple experimental settings.[2-6] Furthermore, anticoagulation reduced the risk of portal vein thrombosis in patients with cirrhosis, and delayed hepatic decompensation in one study.[7] Conversely, mice with a genetically imposed prothrombotic state develop worse liver fibrosis, and patients with hereditary hypercoagulable states show increased risk for development of liver fibrosis.[5,8-10] Thus, there is strong evidence indicating that blood coagulation activity may drive disease pathology in chronic liver injury.

Several mechanisms are proposed to link increased coagulation activity to the development of hepatic fibrosis. Most of these focus on downstream targets of the coagulation proteases factor Xa (FXa) and thrombin. Thrombin catalyzes conversion of soluble fibrinogen to fibrin monomers, which spontaneously polymerize and are then cross-linked by the activated transglutaminase factor XIIIa. Fibrin(ogen) deposits are present in the liver in many experimental settings of hepatic fibrosis,[4,11,12] and it has been proposed that fibrin(ogen) deposition in the injured liver drives local hypoxia and cell death.[13] Although the role of fibrin(ogen) in liver fibrosis is likely context dependent, prior studies indicate that thrombin-catalyzed fibrin polymerization does not contribute to hepatic fibrosis induced by chronic carbon tetrachloride (CCl_4) challenge.[14]

A second mechanism whereby coagulation protease activity may drive hepatic fibrosis is through activation of G-protein–coupled protease-activated receptors (PARs). Whole-body deficiency or pharmacologic inhibition of either PAR-1 or PAR-2 has been shown to reduce experimental hepatic fibrosis in several studies.[15-17] Whereas PAR-1 is the primary receptor for thrombin on human platelets, mouse platelets do not express PAR-1.[18,19] Rather, most studies have focused on PAR-1 expressed by hepatic stellate cells (HSCs), the primary profibrotic cell type in chronic liver disease. HSCs activated in culture conditions express high PAR-1 levels.[15] Recent single-cell RNA sequencing studies revealed increased expression of the gene encoding PAR-1 (*F2R*) in the myofibroblast cell cluster in patients with cirrhosis compared to healthy controls.[20] PAR activation also drives relatively modest profibrotic effects in cultured HSCs.[15,21] Focus on these *in vitro* studies has led to widespread acceptance of direct thrombin-mediated HSC activation in the liver as a critical profibrogenic mechanism. However, the precise role of PAR-1 expressed by HSCs has never been examined *in vivo*.

We generated mice with stellate cell–specific PAR-1 deficiency and used this novel tool to determine the impact of stellate cell–specific PAR-1 deficiency on hepatic fibrosis.

2 | MATERIALS AND METHODS

2.1 | Mice

To generate mice with a stellate cell-specific deletion of PAR-1, female mice expressing *Cre* recombinase driven by the lecithin retinol acyltransferase (LRAT) promoter ($PAR\text{-}1^{+/+}/LRATCre^{pos}$)[22] were crossed with male mice on an identical C57Bl/6J background expressing a conditional flox-flanked PAR-1 allele[23] ($PAR\text{-}1^{flox/flox}/LRATCre^{neg}$). The resulting $PAR\text{-}1^{flox/+}/LRATCre^{pos}$ females were crossed with male $PAR\text{-}1^{flox/+}/LRATCre^{neg}$ mice. The resulting $PAR\text{-}1^{flox/flox}/LRATCre^{pos}$ females were crossed with $PAR\text{-}1^{flox/flox}/LRATCre^{neg}$ males to generate mice for experiments. Male mice were used for these studies in age-matched cohorts between 10 and 19 weeks of age. No mortality was observed over the duration of treatment for any genotype. Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility at Michigan State University at approximately 22 ± 2°C with alternating 12-hour light/dark cycles and were provided *ad libitum* access to reverse-osmosis purified drinking water and rodent diet (Teklad 8940, Envigo, Hackensack, NJ, USA). All animal procedures were approved by Michigan State University (MSU) Institutional Animal Care and Use Committee.

2.2 | Acute and chronic CCl_4 challenge and sample collection

Male $PAR\text{-}1^{flox/flox}/LRATCre$ and $PAR\text{-}1^{flox/flox}$ littermates were challenged with corn oil (vehicle) or 10% CCl_4 in corn oil by

intraperitoneal injection (10 mL/kg) twice weekly (i.e., Tuesday and Friday) for 6 weeks. Three days after the last injection of CCl_4, whole blood was collected from the caudal vena cava under isoflurane anesthesia, and the liver was excised and rinsed in phosphate buffered saline. Blood samples were incubated at room temperature for 30 minutes and then centrifuged at 10 000 *g* for 2 minutes for collection of serum. The left lateral lobe was fixed in 10% neutral-buffered formalin for approximately 96 hours and processed for routine histopathological analysis. Sections of liver from different lobes were snap frozen in liquid nitrogen and saved for other analyses. Chronic CCl_4 exposure was performed in 2 independent experiments with 2 separate cohorts of mice, and results presented include all mice for both experiments. For acute CCl_4 challenge, male PAR-$1^{flox/flox}$/LRATCre and PAR-$1^{flox/flox}$ littermates were challenged with a single dose of 10% CCl_4 in corn oil by intraperitoneal injection (10 mL/kg). Mice were euthanized and samples were collected 48 hours after CCl_4 challenge, as described above.

2.3 | Alpha-naphthylisothiocyanate exposure

Female PAR-$1^{flox/flox}$/LRATCre and PAR-$1^{flox/flox}$ littermates were fed custom diet containing 0.05% α-naphthylisothiocyanate (ANIT) in standard rodent diet (Teklad 8940) formulated by Dyets, Inc (Bethlehem, PA, USA) *ad libitum*.[24] At the end of a 6-week feeding period, mice were euthanized and serum and liver samples were collected as described above.

2.4 | Measurement of serum alanine aminotransferase

Serum alanine aminotransferase (ALT) was measured using commercially available reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.5 | Isolation of primary hepatic stellate cells

Hepatic stellate cells from PAR-$1^{flox/flox}$/LRATCre and PAR-$1^{flox/flox}$ mice were isolated by perfusion/digestion of the liver, as described previously.[25] To determine mRNA expression of the PAR-1 gene (*F2r*), stellate cells were plated overnight in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), then lysed in TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) as per the manufacturer's protocol. Gene expression was measured as outlined below. For experiments involving stimulation of HSCs with PAR-1 agonist peptide or thrombin, primary HSCs isolated from C57Bl6/J mice were cultured in DMEM containing 10% FBS for 72 hours in 12-well tissue culture plates (90 000 cell/well in 1 mL of medium). Medium was then replaced with serum-free medium containing the PAR-1 agonist peptide TFLLR-NH_2 (100 μM, Sigma-Aldrich, St Louis, MO, USA) or human α-thrombin (1 or 10 U/mL, Enzyme Research Laboratories, South Bend, IN, USA) for an additional 24 hours. The concentration of PAR-1 agonist peptide used for these studies is similar to previous studies investigating PAR-1–mediated effects on myofibroblast activation.[15,26-28] Cells were lysed in TRI Reagent, and mRNA was isolated using DirectZol mRNA MiniPrep spin columns (Zymo Research, Irvine, CA, USA). Cell proliferation was measured using a BrdU Cell Proliferation Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions.

2.6 | Histopathology and immunohistochemistry

Hepatic fibrosis was quantified using multiple complementary approaches as described previously.[14] Staining of formalin-fixed paraffin-embedded liver sections to quantify hepatic fibrosis and hepatic stellate cells was performed by the MSU Investigative Histopathology Laboratory, a division of Human Pathology. Briefly, liver sections were stained with Gladstone-modified picrosirius red (PSR) to visualize hepatic fibrosis (i.e., collagen deposits) as described previously.[29] Slides were viewed under polarized light[30] and 10 randomly selected high-powered (400×) images were captured. Total positive staining area was calculated using a batch macro and color deconvolution in ImageJ Fiji.[31] Expression of α-smooth muscle actin (αSMA) protein was detected by immunohistochemical labeling of paraffin-embedded liver tissues as described previously.[29] For quantification of the total area of αSMA staining, slides were first scanned using a Virtual Slide System VS110 (Olympus, Shinjuku, Japan) with a 20× objective. Images were then digitally captured from the entire left lateral lobe (Visiopharm, Broomfield, CO, USA) as described previously.[14] Total area of positive labeling was calculated in an unbiased fashion from approximately 200 images per slide (approximately 200 mm^2 of tissue, captured with a 20× virtual objective) using a batch macro and color deconvolution tool in ImageJ Fiji.[31]

Glial fibrillary acidic protein (GFAP) was detected by immunohistochemical labeling of formalin-fixed paraffin-embedded liver sections. Sections underwent enzyme-induced epitope retrieval using 0.03% Pronase E (Sigma-Aldrich), followed by blocking of endogenous peroxidase with 3% hydrogen peroxide/methanol bath for 30 minutes. Labeling was performed at room temperature on the IntelliPath Flex Autostainer (Biocare Medical, Pacheco, CA, USA). After blocking for nonspecific protein with Rodent Block M (Biocare Medical) for 20 minutes, sections were incubated with a polyclonal rabbit anti-cow GFAP (Agilent Technologies, Santa Clara, CA, USA; #Z0334) in normal antibody diluent (Scytek Laboratories, Logan, UT, USA) for 1 hour. Micro-Polymer (Biocare Medical) reagents were subsequently applied for 30 minutes followed by reaction development with IntelliPath Flex Warp Red (Biocare Medical) and counterstained with hematoxylin. To quantify the number of GFAP-positive cells, slides were scanned using a Virtual Slide System VS110, and the number of GFAP-positive cells was counted using the cell counter function in ImageJ Fiji in 15 randomly selected fields (captured with a 10× virtual objective in OlyVIA software, Olympus).

For assessment of chronic liver injury, paraffin-embedded liver sections were stained with hematoxylin and eosin and examined by a board-certified veterinary pathologist (KJW) in a blinded fashion. Quantification of hepatocellular necrosis in the entire left lateral lobe in response to acute CCl_4 challenge was performed using hematoxylin and eosin–stained liver sections as described previously.[32]

Collagen type I was detected by immunofluorescent labeling of frozen liver sections as described previously.[14] Slides were scanned using Virtual Slide System VS110, and the area of positive labeling was quantified in 4-5 randomly selected images (captured with a 4× virtual objective in OlyVIA software, approximately 40 mm^2 of liver tissue) using a threshold analysis tool in ImageJ Fiji.

2.7 | RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from snap-frozen liver or isolated HSCs using TRI Reagent according to the manufacturer's protocol (Molecular Research Center). One microgram of total RNA was used to synthesize cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Rockville, MD, USA) and a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). SYBR Green quantitative real-time PCR amplification was performed using a CFX Connect thermal cycler (Bio-Rad Laboratories) with primers purchased from IDT and PerfeCTa Sybr Green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). The expression of each gene was normalized to the geometric mean threshold cycle (Ct) of individual housekeeping genes, *Hprt* and *Gapdh*, and relative fold change was determined using the ΔΔCt method. Primer sequences used are previously described.[11]

2.8 | Statistical analysis

Statistical significance was determined using the Student's *t* test or a 2-way analysis of variance with the Student-Newman-Keuls post hoc test, as appropriate. Differences were considered significant at $P < 0.05$.

3 | RESULTS

3.1 | Efficient deletion of PAR-1 in hepatic stellate cells isolated from PAR-$1^{flox/flox}$/LRATCre mice

The first objective of this study was to generate mice with a stellate cell-specific deletion of PAR-1. To measure deletion of stellate cell PAR-1, HSCs were isolated from unchallenged PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice as described in Materials and Methods. PAR-1 gene (*F2r*) expression was reduced by > 95% in PAR-$1^{flox/flox}$/LRATCre mice (Figure 1A). Next, we sought to confirm that stellate cell-specific PAR-1 deletion did not impact the number of HSCs in vehicle (corn oil)–treated mice. Stellate cells were detected by immunofluorescent labeling of the hepatic stellate cell marker GFAP in liver sections (Figure 1B,C). The number of red punctate GFAP-positive cells was equivalent in PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice, indicating that deletion of PAR-1 did not impact the number of HSCs in unchallenged mice.

3.2 | Stellate cell–specific PAR-1 deletion does not impact acute CCl_4-induced hepatotoxicity

The acute response to CCl_4 includes hepatotoxicity typified by centrilobular necrosis, inflammation, and stellate cell activation.[25,33] Chronic liver damage induced by repetitive CCl_4 challenge provokes lasting activation of hepatic stellate cells and excess deposition of collagen (ie, fibrosis). Mechanisms specific to the manifestation of hepatic fibrosis are best identified after assuring the hepatotoxic response to CCl_4 is not affected by the experimental intervention. Thus, we first determined the impact of stellate cell PAR-1 deficiency on the hepatotoxic response after acute CCl_4 challenge. PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice were challenged with a single dose of CCl_4 or vehicle, and tissues were collected for analysis 48 hours later. Acute CCl_4 challenge caused marked centrilobular hepatocellular necrosis, as indicated by elevated serum ALT levels and area of centrilobular necrosis in the left lateral lobe (Figure 2A-C). Serum ALT levels and hepatocellular necrosis were equivalent in CCl_4-challenged PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice, indicating that stellate cell–specific PAR-1 deficiency did not impact liver injury in response to acute CCl_4 challenge. Acute CCl_4 challenge also causes transient activation of HSCs.[25] The extent of stellate cell activation after acute CCl_4 challenge was equivalent in PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice, as indicated by αSMA labeling and expression αSMA (*Acta2*) and collagen type I (*Col1a1*, Figure 2D-F) mRNAs. Thus, stellate cell PAR-1 deficiency does not affect the acute hepatotoxic response after CCl_4 challenge.

3.3 | Stellate cell–specific PAR-1 deletion reduces HSC activation and improves injury resolution following chronic CCl_4 challenge

To determine the role of stellate cell PAR-1 in hepatic fibrosis, PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice were challenged for 6 weeks with CCl_4 or vehicle as described in Materials and Methods. Chronic CCl_4 challenge caused marked stellate cell activation in PAR-$1^{flox/flox}$ mice, as indicated by increased αSMA labeling and mRNA induction of *Col1a1* compared to vehicle-treated mice (Figure 3A-C). Chronic CCl_4 challenge also induced hepatic expression of the profibrotic mediators transforming growth factor beta-1 (*Tgfb1*) and tissue inhibitor of metalloproteinase-1 (*Timp1*) in PAR1$^{flox/flox}$ mice (Figure 3D,E). Stellate cell-specific PAR-1 deletion caused a significant reduction in CCl_4-induced αSMA labeling and *Col1a1* mRNA expression (Figure 3A-C), indicating that stellate cell–specific PAR1 deletion reduces HSC activation.

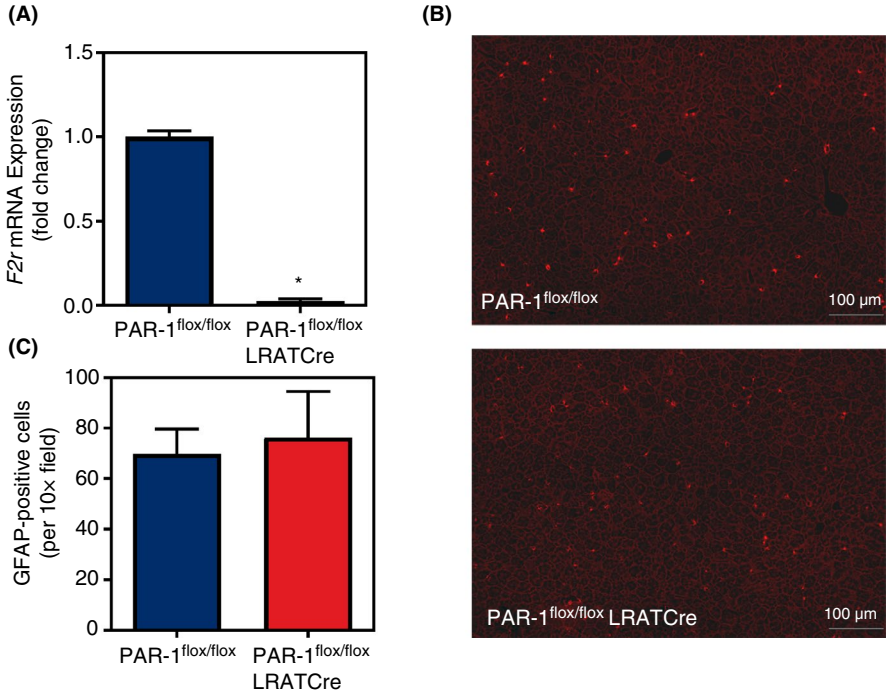

FIGURE 1 Efficient deletion of protease-activated receptor-1 (PAR-1) gene (*F2r*) in PAR1$^{flox/flox}$/LRATCre stellate cells with no effect on baseline stellate cell number. (A) Hepatic stellate cells were isolated from PAR-1$^{flox/flox}$ and PAR-1$^{flox/flox}$/LRATCre mice as described in Materials and Methods. Expression of PAR-1 (*F2r*) was measured by quantitative real-time PCR (n = 3 mice per genotype). (B) Representative photomicrographs (10× virtual magnification) of glial fibrillary acidic protein (GFAP) labeling (red) in paraffin-embedded liver sections from vehicle-treated mice (n = 5 mice per genotype). (C) Quantification of the average number of GFAP-positive cells counted per 10× field in 15 randomly selected fields. Data are presented as mean + SEM. *$P < .05$ compared to PAR-1$^{flox/flox}$ mice

Carbon tetrachloride-induced expression of *Tgfb1* and *Timp1* was not affected by stellate cell-specific PAR1 deletion (3D-E).

Previous studies have reported that activation of PAR-1 promotes a profibrotic phenotype in cultured stellate cells.[6,15,21,34] Having determined that stellate cell-specific PAR-1 deletion reduces HSC activation and collagen deposition in response to chronic CCl_4 challenge, we measured induction of profibrotic mediators in wild-type stellate cells activated in culture for three days before treatment with PAR-1 agonists. These cells expressed high levels of PAR-1 (*F2r*) mRNA (not shown). We were surprised to observe that stimulation of wild-type primary HSCs with PAR-1 agonist peptide (TFLLR-NH_2) or thrombin had only modest effects on induction of αSMA (*Acta2*, Figure S1A). Furthermore, PAR-1 activation did not cause an induction of *Col1a1* mRNA or stimulate HSC proliferation (Figure S1B,C). Thus, while direct activation of PAR-1 may contribute to stellate cell activation, we also considered alternative mechanisms through broad assessment of pathological changes occurring in the liver after chronic CCl_4 challenge. Liver sections from PAR-1$^{flox/flox}$ and PAR-1$^{flox/flox}$/LRATCre mice were examined in a masked fashion by a veterinary pathologist as described in Materials and Methods. As anticipated, vehicle-treated PAR-1$^{flox/flox}$ and PAR-1$^{flox/flox}$/LRATCre mice displayed normal hepatic architecture (Figure 4A). Chronic CCl_4 challenge in PAR-1$^{flox/flox}$ mice caused marked widespread hepatocellular swelling and cytoplasmic vacuolation accompanied by hepatocellular necrosis (Figure 4A, arrow) and frequent enlarged centrilobular hepatocytes with mitotic figures (Figure 4A, arrowhead). Interestingly, each of these histological features was reduced in CCl_4-challenged PAR-1$^{flox/flox}$/LRATCre mice, including a reduced area of residual hepatic necrosis (Figure 4A). Consistent with our findings after acute CCl_4 challenge (Figure 2), changes in serum ALT reflected a similar hepatotoxic response in PAR-1$^{flox/flox}$ and PAR-1$^{flox/flox}$/LRATCre mice after chronic CCl_4 challenge (Figure 4B). Thus, whereas increased serum ALT levels suggest an equivalent initial hepatotoxic response, stellate cell PAR-1 deficiency improved resolution of liver pathology after CCl_4 challenge.

3.4 | Stellate cell–specific PAR-1 deletion reduces CCl_4-induced hepatic collagen deposition

Chronic stellate cell activation induced by unresolved liver injury and/or persistent exposure to various activators leads to hepatic fibrosis characterized by deposition of collagen in the liver. Collagen deposition was analyzed by two independent methods, including quantification of PSR staining and specific collagen type 1 immunolabeling. PSR is a chemical stain that, when examined under polarized light, isolates the birefringent property of type I and III collagen.[30,35] Chronic CCl_4 challenge increased the area of PSR staining in both PAR-1$^{flox/flox}$ and PAR-1$^{flox/flox}$/LRATCre mice compared to vehicle-treated mice (Figure 5A,C). Increased CCl_4-induced collagen deposition was further confirmed by immunolabeling of type 1 collagen (Figure 5B,D). In agreement with reduced stellate cell activation and *Col1a1* mRNA expression in CCl_4-challenged PAR-1$^{flox/flox}$/LRATCre mice, stellate cell-specific PAR-1 deficiency significantly reduced hepatic collagen deposition in CCl_4-challenged animals (Figure 5A-D). Taken together, these results indicate that stellate cell PAR-1 contributes to CCl_4-induced liver fibrosis.

4 | DISCUSSION

Multiple studies have proposed that the mechanism whereby coagulation proteases drive liver fibrosis is via activation of PARs. Indeed,

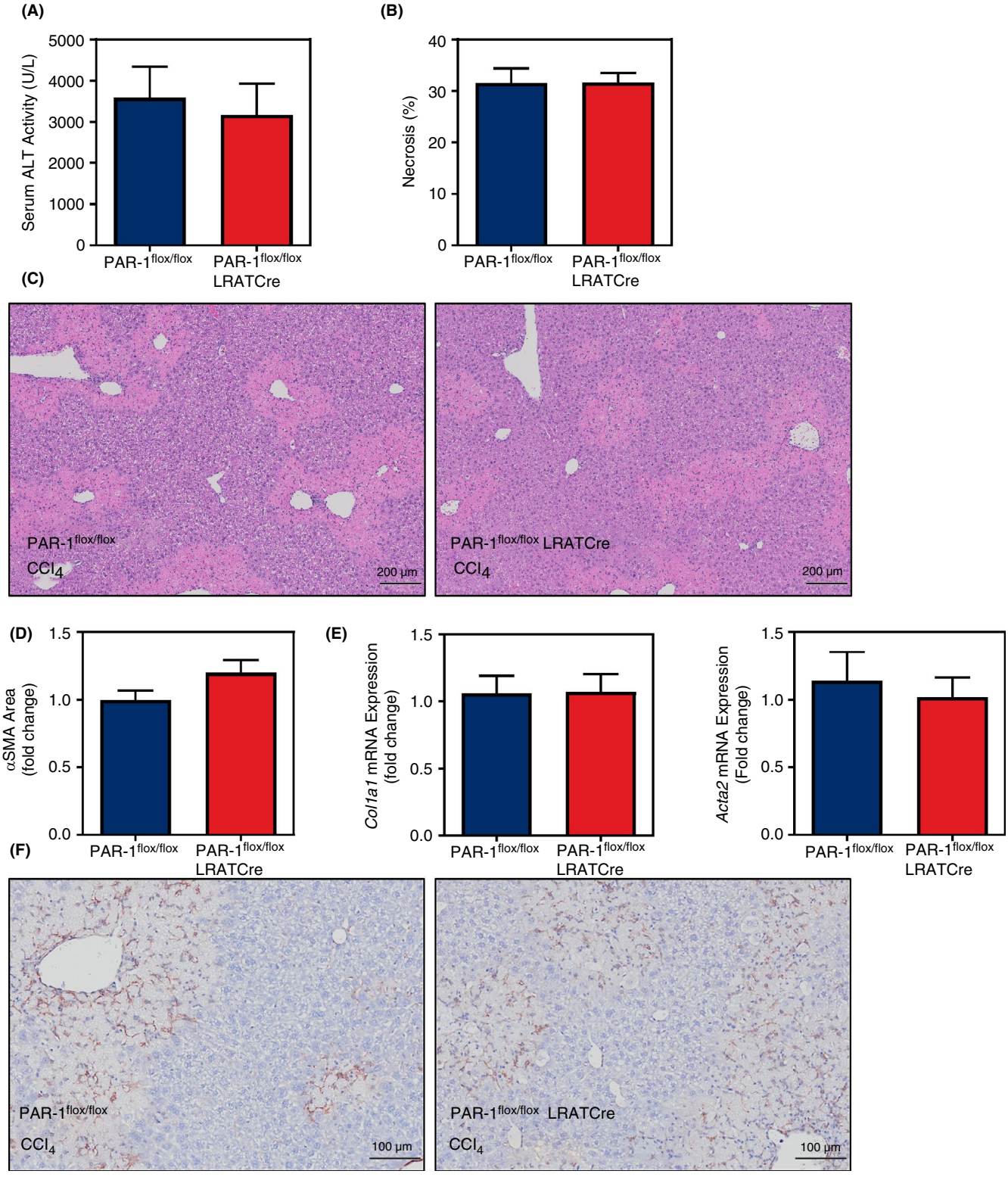

FIGURE 2 Effect of stellate cell–specific protease-activated receptor-1 (PAR-1) deletion on acute carbon tetrachloride (CCl_4)-induced liver injury and stellate cell activation. PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice were challenged with a single injection of CCl_4, and hepatocellular necrosis and stellate cell activation were assessed 48 hours after injection. Hepatocellular necrosis was measured via (A) serum alanine aminotransferase (ALT) levels and (B) quantification of the area of necrosis in hematoxylin and eosin (H&E)–stained liver sections as described in Materials and Methods. (C) Representative photomicrographs (4× virtual magnification) of H&E-stained paraffin-embedded liver sections. Stellate cell activation was assessed by (D) quantification of the area of α-smooth muscle actin (αSMA) labeling in liver sections and mRNA induction of (E) *Col1a1* and *Acta2* by quantitative real-time PCR in liver homogenates. (F) Representative photomicrographs (10× virtual magnification) of αSMA immunolabeling of paraffin-embedded livers. Data are presented as mean + SEM (n = 8 mice per group)

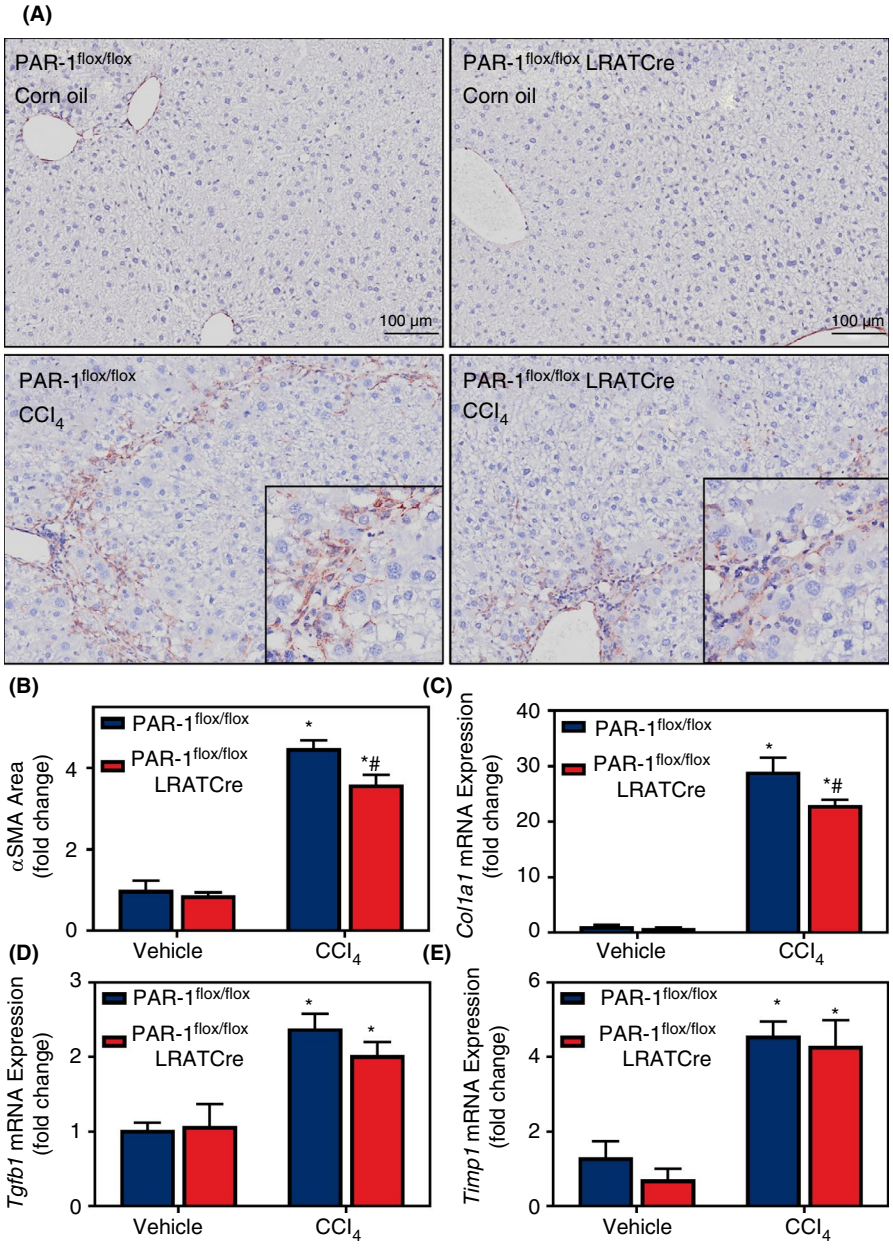

FIGURE 3 Effect of stellate cell-specific protease-activated receptor-1 (PAR-1) deletion on chronic carbon tetrachloride (CCl_4)-induced stellate cell activation and induction of profibrotic mediators. PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice were challenged with CCl_4 or vehicle (corn oil) for 6 weeks, and stellate cell activation was assessed 3 days after the last injection. (A) Representative photomicrographs (10× virtual magnification, 20× virtual magnification inset) of αSMA immunolabeling of paraffin-embedded livers. (B) Area of αSMA immunolabeling was quantified as described in Materials and Methods (n = 4-8 mice per group). Gene induction of *Col1a1* (C), *Tgfb1* (D), and *Timp1* (E) were measured by quantitative real-time PCR in whole liver homogenates (n = 4-15 mice per group). Data are presented as mean + SEM. $^{*}P < .05$ compared to vehicle-treated mice of the same genotype, $^{\#}P < .05$ compared to CCl_4-challenged PAR-$1^{flox/flox}$ mice

activation of PAR-1 expressed by HSCs is frequently depicted as the final step in pathways connecting coagulation to hepatic fibrosis, yet this hypothesis has been untested. Although depicted as a conclusive element of schema in numerous review articles,[1,36,37] this mechanism has simply been inferred based on 3 independent observations, including the reduction of experimentally induced liver fibrosis in PAR-$1^{-/-}$ mice and in mice treated with PAR-1 antagonists,[15-17,38] evidence that PAR-1 agonists induce activation of hepatic stellate cell lines in culture,[6,15,21,34] and the reduction in hepatic fibrosis evident after administration of anticoagulant drugs.[2,4-6,34,39] The current study capitalizes on the first mouse model of hepatic stellate cell–specific PAR-1 deficiency to determine the role of stellate cell PAR-1 in liver pathobiology. Prior studies have demonstrated that global PAR-1 deficiency reduces CCl_4-induced hepatic fibrosis by approximately 50%.[16] Our results indicate that stellate cell–specific PAR-1 deletion reduces hepatic collagen deposition by approximately 35% (Figure 5D), providing the first definitive evidence for the role of stellate cell PAR-1 as a major profibrotic pathway.

PAR-1 activation may indeed participate in the conversion of quiescent hepatic stellate cells to myofibroblast-like, collagen-expressing cells. Stimulation of the rat HSC line HSC-T6 with thrombin increases mRNA expression of *Col1a1* and induces stellate cell proliferation and contractility,[15] and has been shown in other studies to modestly increase expression of *Acta2*.[34] Similar results have been observed when activated primary rat HSCs are stimulated with thrombin.[21] Stimulation of the human HSC line LX-2 with thrombin with or without FXa promotes altered HSC morphology and modestly induces expression of genes encoding profibrotic mediators, including *Acta2* and *Col1a1*.[6,34] Stimulation of HSC-T6 cells and primary rat HSCs with the synthetic agonist peptide SFLLR-NH_2 had similar effects,[15,21] although this agonist peptide also activates PAR-2.[40] Our *in vitro* studies using primary stellate cells isolated from

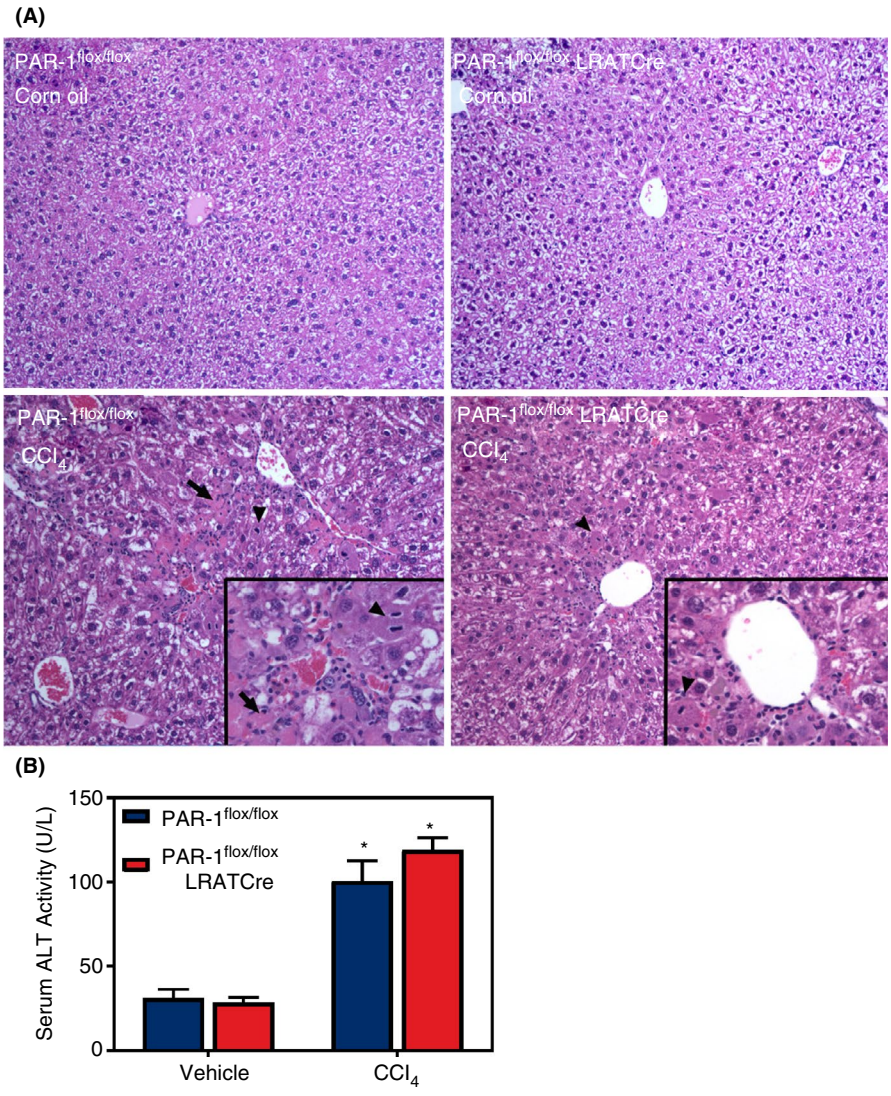

FIGURE 4 4 Effect of stellate cell–specific protease-activated receptor-1 (PAR-1) deletion on chronic carbon tetrachloride (CCl_4)-induced liver injury. PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice were challenged with CCl_4 or vehicle (corn oil) for 6 weeks, and liver injury was assessed 3 days after the last injection. (A) Representative photomicrographs (100× magnification, 200× magnification inset) of hematoxylin and eosin–stained paraffin embedded liver sections examined by a board-certified veterinary pathologist (n = 4 mice per group). Arrows indicate areas of acute hepatocellular necrosis, and arrowheads indicate enlarged hepatocytes with mitotic figures. (B) Serum alanine aminotransferase levels were measured in vehicle- and CCl_4-challenged mice (n = 4-15 mice per group). Data are presented as mean + SEM. *$P < .05$ compared to vehicle-treated mice of the same genotype

mice largely agree with these findings, indicating that stimulation of primary mouse HSCs with thrombin or the PAR1-specific agonist peptide TFLLR-NH_2 produces modest induction of *Acta2* expression (Figure S1A). Additionally, in agreement with previous studies,[6] we found that stimulation of HSCs with PAR-1 agonists modestly reduced induction of *Col1a1* (Figure S1B). We did not observe an effect of PAR-1 agonist peptide on stellate cell proliferation (Figure S1C). This could be explained by differences in the *in vitro* model used (ie, cultured cell line vs primary cells) and/or the sequence of PAR agonist peptide. Indeed, effects (or lack thereof) in culture conditions of stellate cells should be interpreted cautiously, as *in vitro* settings may not adequately recapitulate *in vivo* HSC activation. Several studies suggest that conditions imposed by cell culture, including adherence to a firm plastic surface, imposes significant barriers to understanding the complexities of stellate cell activation in the injured liver.[41] It is also possible that PAR-1 activation is necessary, but not sufficient for stellate cell activation. Other profibrotic signals present in the liver, such as TGF-β, or additional PAR-1 agonists not present in an *in vitro* system may be required for PAR-1–mediated stellate cell activation *in vivo*. These challenges emphasize the importance of our observations using PAR-$1^{flox/flox}$/LRATCre mice and highlight the need for novel cell culture models. Pairing 3-dimensional and coculture methods with assessment of stellate cell activation *in vivo* may be the preferred method to further define the precise contribution of PAR-1 to stellate cell activation.

The absence of PAR-1 on mouse platelets[18,19] affords unique opportunity to define PAR-1–dependent disease pathogenesis outside its primary hemostatic function. Although PAR-1 may serve simply as a trigger for profibrogenic stellate cell activity, we also observed that stellate cell PAR-1 deficiency improved resolution of hepatic injury without impacting the initial acute hepatotoxic response to CCl_4. Critically, this suggests that the role of stellate cell PAR-1 may encompass multiple mechanisms. By way of example, stellate cells also command the inflammatory response through receipt and expression of chemokines and their receptors. Notably, hepatic infiltration of T lymphocytes is significantly reduced in mice with whole-body PAR-1 deficiency after chronic CCl_4 challenge.[16] Interestingly, expression of profibrotic mediators which are predominantly expressed by inflammatory cells (eg, *Tgfb1* and *Timp1*), was unaffected by stellate cell–specific

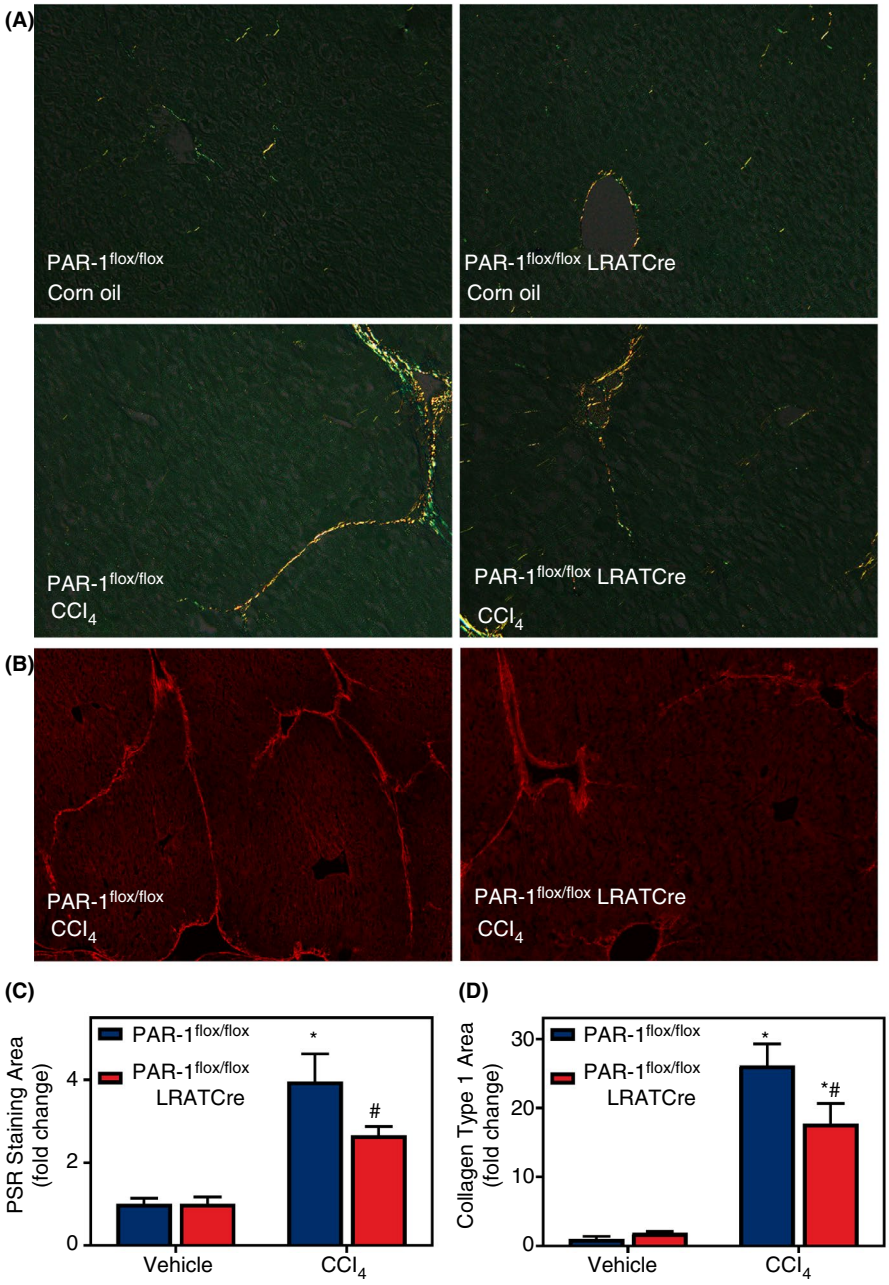

FIGURE 5 5 Effect of stellate cell-specific protease-activated receptor-1 (PAR-1) deletion on chronic carbon tetrachloride (CCl_4)-induced hepatic fibrosis. PAR-$1^{flox/flox}$ and PAR-$1^{flox/flox}$/LRATCre mice were challenged with CCl_4 or vehicle (corn oil) for 6 weeks, and hepatic collagen deposition was assessed 3 days after the last injection. (A) Representative photomicrographs (200× magnification) of picrosirius red (PSR)-stained paraffin-embedded liver sections viewed under polarized light. (B) Representative photomicrographs (5× virtual magnification) of collagen type I immunolabeling of frozen liver sections. Positive area of PSR (C) and collagen type I (D) was quantified as described in Materials and Methods. Data are presented as mean + SEM (n = 4-10 mice per group). *P < .05 compared to vehicle-treated mice of the same genotype. #P < .05 compared to CCl_4-challenged PAR-$1^{flox/flox}$ mice

PAR-1 deletion. While the focus has been on PAR-1–mediated stellate cell profibrotic activity, our analysis of liver histopathology suggests a more complex mechanism in which PAR-1–dependent stellate cell activation impairs injury resolution, ultimately perpetuating a state where collagen deposition occurs in place of normal liver repair.

Notably, PAR-1 is expressed by multiple hepatic nonparenchymal cells and other cells infiltrating the injured liver. These include a variety of inflammatory cells (eg, monocytes, neutrophils), resident liver macrophage (ie, Kupffer cells), and various lymphocyte populations.[42-44] Global PAR-1 deficiency also reduced hepatic inflammation in 2 different models of nonalcoholic steatohepatitis.[45] In addition, mice with PAR-1–deficient hematopoietic cells displayed reduced hepatic fibrosis after chronic CCl_4 challenge.[43] It seems plausible that PAR-1 on monocytes/macrophages may contribute to the development of hepatic fibrosis. Another subset of hematopoietic cells that may contribute to hepatic fibrosis are fibrocytes,[46] and interestingly, one study suggested that thrombin stimulation promotes fibrocyte differentiation.[47] However, it is worth noting that LRATCre-positive HSCs that contribute to the vast majority of the profibrogenic response are not bone marrow derived.[22] Thus, while PAR-1 expressed by HSCs appears to play an important role in hepatic fibrosis, a role for PAR-1 expressed by other cell types in this complex pathology should not be excluded. Furthermore, the role of stellate cell PAR-1 may be different based on the etiology of the liver disease. Interestingly, we found that stellate cell PAR-1 did not contribute to hepatic injury or fibrosis in experimental biliary fibrosis induced by exposure to the biliary toxicant ANIT (Figure S2). Importantly, the contribution of PAR-1 to fibrosis in this setting[17] may be driven by other cell types,

including portal fibroblasts and biliary epithelial cells.[48] Therefore, it is important to note that the role of stellate cell PAR-1 in hepatic fibrosis may be context specific.

Strong evidence indicates a contribution of PAR-1 to liver pathology in multiple experimental settings.[15-17,45] Primary attention has been on thrombin as a PAR-1 agonist primarily because administration of direct thrombin inhibitors reduces hepatic stellate cell activation and hepatic fibrosis in various experimental settings.[6,34] Moreover, low-molecular-weight heparins,[2,3] warfarin,[5] and the FXa inhibitor rivaroxaban[4] have also been shown to reduce HSC activation and experimental hepatic fibrosis. However, there is little to no definitive evidence that thrombin is the PAR-1 agonist driving hepatic fibrosis. For example, several studies have also shown that PAR-2 contributes to stellate cell activation and drives hepatic fibrosis.[49,50] PAR-1 activation may in fact directly contribute to PAR-2 activation through transactivation. These receptors exist in close proximity on the cell surface, and the activation peptide generated by thrombin cleavage of PAR-1 also activates PAR-2.[51] Additionally, these 2 receptors have been shown to exist in an active heterodimer.[50] Anticoagulant drugs may directly or indirectly reduce PAR-2 activation by coagulation factor Xa.[4,52] Furthermore, PAR-1 is activated by a wide variety of proteases participating chronic liver injury, including other coagulation factors (e.g., factor VIIa and FXa), neutrophil elastase, matrix metalloproteinases, plasmin, and activated protein C (APC).[52] Various proteases may activate PAR-1 through cleavage at different residues in the extracellular domain. For example, APC cleaves PAR-1 at Arg46, whereas thrombin cleaves PAR-1 at Arg41. PAR-1 activation by these 2 different ligands activates different downstream signaling pathways, which ultimately control disease phenotype in experimental sepsis and stroke.[53] Future studies should investigate differential activation of hepatic stellate cells by these 2 different PAR-1 agonists. Finally, there is also a need to better evaluate intrahepatic thrombin activity in the injured liver. Whereas numerous studies use intrahepatic fibrin(ogen) deposition as an index of hepatic thrombin activity, our recent studies indicate this may not be a reliable indicator of intrahepatic coagulation.[14] Overall, it will be important to decipher the PAR-1 agonist driving hepatic stellate cell activation in liver disease, particularly because different PAR-1 ligands initiate different downstream signaling pathways, which can impact disease pathology.[53,54]

In conclusion, we document for the first time that PAR-1 expressed by HSCs drives CCl_4-induced liver fibrosis *in vivo*. These definitive studies solidify a long-standing but untested hypothesis that stellate cell PAR-1 contributes to liver fibrosis. Notably, the bleeding risk associated with platelet PAR-1 inhibition may limit the utility of systemic delivery of small molecule PAR-1 antagonists (ie, vorapaxar) in patients with liver disease. However, modifying PAR-1 expression (eg, siRNA) or PAR-1 activity (eg, pepducins[55]) selectively in the liver or specifically in stellate cells may have antifibrotic effects without imposing a bleeding risk. Overall, the novel studies herein provide a capstone to the hypothesis that stellate cell PAR-1 drives hepatic fibrosis and provide further evidence in support of PAR-1-targeted therapies as putative antifibrotics.

ACKNOWLEDGMENTS

The authors thank Dr. Robert Schwabe (Columbia University Irving Medical Center) for generously providing the LRATCre mice used to generate mice for these studies.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

LGP contributed to all aspects including conception and design of experiments, acquisition, analysis, and interpretation of data, and drafting of the manuscript. AP and HMC-F acquired and analyzed data. KW, BLC, and JSP interpreted data and contributed to drafting of the manuscript. JPL contributed to all aspect of the project and assisted in drafting of the manuscript. All authors read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Poole LG, Pant A, Cline-Fedewa HM, et al. Liver fibrosis is driven by protease-activated receptor-1 expressed by hepatic stellate cells in experimental chronic liver injury. *Res Pract Thromb Haemost*. 2020;4:906–917. https://doi.org/10.1002/rth2.12403